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In training and in combat, soldiers are under the constant threat of injury. Injury that results in tissue necrosis can activate the immune system and ultimately enhance disturbances in organ function. Knowledge of the immune activation mechanisms may lead to methods of reducing tissue damage. Mechanistic studies require precise control over the complex factors that coordinate the tissue injury cascade. An in vitro model would provide such a level of control. However, the feasibility of obtaining the required immune system tissues, such as human whole blood (HWB), endothelial cells and keratinocytes from the same human donor is unlikely. As such, an in vitro model comprised of tissues from immunologically distinct donors is perhaps the next best approach, but this necessitates an experimental design that controls for adverse histocompatibility or tissue rejection reactions. The current study employed permeable membranes to physically separated immunologically distinct immune system tissues. This permitted tissue intercommunication via soluble mediators, while placing a barrier between the immunologically distinct tissues obviated tissue rejection, since contact with the histocompatibility complexes on their surfaces was prevented. Using such an approach, the capacity to mount immune responses following tissue injury like that induced by freeze/thaw tissue damage (i.e., necrosis) was evaluated. Findings suggested the in vitro conditions within this system could not support immune activation of HWB via differences in tissue histocompatibility (i.e., direct HWB exposure to intact tissues) or necrosis (i.e., HWB exposure to soluble mediators of freeze/thaw-damaged tissues). This outcome provided evidence to suggest initiation of immune responses induced by tissue necrosis or rejection required input from secondary lymphoid tissues (i.e., lymph nodes, spleen, etc.) and specialized antigen-presenting cells, which were absent in the present experimental design.

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EVALUATION OF AN IN VITRO MODEL OF HUMAN IMMUNE ACTIVATION INDUCED BY FREEZE/THAW TISSUE DAMAGE

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BACKGROUND

Heat, cold, high terrestrial altitude, strenuous exercise and vesicant exposure are environmental factors that the war fighter may encounter on the battlefield. The activation and resulting actions of the immune system contribute to the tissue injury induced by such environmental conditions. Presently, a model that permits control over the complex factors leading to local inflammatory reactions, which can employ human tissues from unrelated donors, while avoiding issues of histocompatibility is lacking. Such a model may allow the study of human immune responses mediated by tissue injury and necrosis. This would contribute to our understanding of pathophysiological mechanisms exacerbating tissue injury induced by immune activation and assist in the identification of ameliorative measures to block or abate this injury cascade. The present study evaluated an *in vitro* approach to model local immune responses induced by freeze/thaw-damaged tissue.

EXECUTIVE SUMMARY

In training and in combat, a constant threat to the soldier is the risk of injury. Injury that results in tissue necrosis can activate local inflammation, which enhances disturbances in tissue and organ function. Knowledge of the mechanisms that lead to such injury may provide methods to prevent, reduce or ameliorate the immune system contribution to tissue damage. Mechanistic studies require precise control over the numerous and complex factors that coordinate the tissue injury cascade. An *in vitro* model would provide such a level of control and permit study of local immune responses. However, the feasibility of obtaining the required immune system tissues, such as human whole blood (HWB), endothelial cells (EC) and keratinocytes (KC) from the same human donor is unlikely. As such, an *in vitro* model comprised of human immune system tissues from immunologically distinct donors is perhaps the next best approach, but this necessitates the incorporation into the experimental design a means of controlling for adverse histocompatibility or tissue rejection reactions. The current study employed permeable membranes to physically separated immunologically distinct immune system tissues. This design permitted tissue intercommunication via soluble mediators, while tissue rejection was obviated by placing a barrier between the immunologically distinct tissues, such that contact with the histocompatibility complexes on their surfaces was prevented. Using such an approach, the capacity to mount immune responses following tissue injury like that induced by freeze/thaw tissue damage (i.e., necrosis) was evaluated. Findings suggested the *in vitro* conditions within this system could not support immune activation of HWB via differences in histocompatibility (i.e., direct HWB exposure to intact tissues) or necrosis (i.e., HWB exposure to soluble mediators of freeze/thaw-damaged tissues). This outcome provided evidence to suggest initiation of immune responses induced by adverse histocompatibility reactions or tissue necrosis required input from secondary lymphoid tissues (i.e., lymph nodes, spleen, etc.) and specialized dendritic or antigen presenting cells, which were absent in the present experimental design.

INTRODUCTION

An inflammatory process and/or the presence of immune mediators, cytokines have been described in cases of human heatstroke (1,2,3), as well as, in frostbite damage (4,5), high altitude pulmonary edema (6,7) and injuries associated with exposure to chemical vesicants (8,9) or strenuous exercise (10-13). These are all injuries that can have significant impact on the conduct of military operations.

Defining the pathophysiology that contributes to immune activation requires study under conditions in which severe tissue damage is induced. Moreover, the experimental design must adequately control for exogenous (bacterial endotoxins) and endogenous (tissue necrosis) factors that influence an immune response after tissue injury. Studies under such conditions could reveal mechanisms of injury that can lead to preventive or treatment approaches for the amelioration of injurious immune responses.

Appropriately controlled studies of severe tissue injury are limited by the ethical considerations necessary for the protection of the health and well being of human test subjects. To support studies of severe forms of injury, animal or *in vitro* models are generally employed. In the present environment that supports alternatives to reduce the suffering and use of animals in research (14), the development of new animal models is contraindicated. Moreover, relative to immune studies, commercial test systems for the numerous inflammatory mediators that are in need of study are not as readily available for non-human species and controlling for exogenous immune activators (i.e., endotoxins) in an animal model would be as challenging as studies conducted in humans. Another confounding issue is the limitation to systemic sampling when employing animal or human subjects. This does not lend itself to immune activation studies, since many mediators are paracrine in nature. As such, they act across short distances and may only be found in the local inflammatory environment (15). Furthermore, since immune mediators can have opposite local and systemic effects (16), what is noted systemically may have little relevance to actions occurring in the

local environment. An *in vitro* system for the study of severe tissue injury is desirable, since it can employ human tissues, separate endogenous from exogenous contributions and permit study of local inflammatory responses in the immune activation cascade.

Differences in tissue type of an *in vitro* model can influence the magnitude of release of immune modulators (i.e., prostaglandin) after heat injury (17). This illustrates the importance of having the appropriate tissues in communication with each other in order to model appropriately immune function. An *in vitro* system should be comprised of whole blood, EC and organ tissue to approximate the *in vivo* state, since these are the elements intimately involved in immune responses after injury. There are examples of *in vitro* systems for the study of immune responses that combine vascular, conduit and organ tissues. However, these systems are generally designed to study allograft rejection and as such, differences in tissue histocompatibility are an integral feature in such models (18-20). Since differences in histocompatibility may themselves induce an immune response, the use of immunologically distinct tissues may compromise a model intended for the study of immune activation induced by tissue necrosis.

The present study evaluated an *in vitro* approach for the study of immune activation after freeze/thaw damage (i.e., tissue necrosis). The system was comprised of HWB, EC and KC from immunologically distinct sources. To obviate histocompatibility issues, some experiments employed permeable membranes of various pore sizes to physically separate the cell surface histocompatibility complexes of intact or freeze/thaw-damaged tissues (EC and/or KC), however exchange of soluble mediators through the membrane pores to support communication among the tissues was retained. Other experiments permitted direct interaction among the various intact tissues. Signs of immune activation were monitored by analysis of interleukin-4 and 12 (IL-4 and IL-12, respectively), cytokines that respectively regulate either humoral or cellular immune responses (21, 22-24). Other studies explored for signs of a proliferation response by immune cells. It was hypothesized, absence of an immune response when intact, immunologically distinct tissues were physically separated from

each other, while its presence when soluble mediators from freeze/thaw-damaged tissues passed through the small pores (0.4μ) of a permeable membrane would be evidence for immune activation by a process of tissue necrosis.

METHODS

Male blood samples from 3 military volunteers were studied, after they had given informed consent and a medical monitor had ascertained they were free of disease or other obvious factors that might compromise the goals of the study. These volunteers were between the ages of 18 and 35 years. They did not use tobacco products. Following an overnight fast (no food after 2000 hrs) and before consumption of any food the next morning, each volunteer had a heparinized (40 U/ml) blood sample (200 ml) collected via vena puncture.

Human, adult, aortic EC and human, adult KC were obtained from Clonetics® normal human cell systems of San Diego, CA. To construct a barrier between the human tissues, EC and KC were cultured on the surfaces of permeable membranes (0.4 and/or 3.0μ), which were inserted into the wells of a Transwell® tray (Costar; Corning, NY) containing heparinized HWB, as depicted in Figure 1. All cell seedings employed 40,000 cells/ml, culture media and conditions as prescribed by the vendor (Clonetics®, San Diego, CA). For studies employing intact cells, seedings were performed 72 hrs prior to experimentation. Cell confluency on the various surfaces was between 75 and 90% at the time of experimentation. For studies employing freeze/thaw-damaged cells, procedures as described by the vendor (Clonetics®, San Diego, CA) for detaching cells from tissue culture surfaces using trypsin were followed. The number of detached cells was adjusted to 40,000 cells/ml. These cell suspensions were placed at –20°C and maintained in a frozen state until thawed at 37°C just prior to time of experimentation.

Figure 2a illustrates the experimental conditions for immune responses induced by intact EC or KC exposed to HWB or unsupplemented culture medium. Conditions

similar to that noted *in vivo* were created by exposing intact EC to HWB, while a 0.4μ membrane separated intact KC (Figure 2b). Figure 2c illustrates the experimental conditions for the negative control in which HWB was cultured in the absence of any other tissues. To simulate a state of tissue necrosis, soluble mediators from immunologically distinct, freeze/thaw-damaged EC and/or KC were exposed to HWB via the pores of 3.0 or 0.4μ membranes (Fig. 2d). Positive controls for immune responses consisted of HWB exposed to *Escherichia coli* endotoxin 0111:B4 (100 μg/ml) as depicted in Figure 2e. All experimental conditions were incubated under rocking conditions in a 5% CO_2 incubator at 37°C. Blood plasma or culture media was collected after 6, 24 and 48 hrs of incubation.

Standard enzyme-link immunosorbant assay (ELISA) technology (R&D Systems, Minneapolis, MN) was employed to quantitate IL-4 and IL-12 cytokine expression. Comparison of the cytokine expressions over the 48 h test period for the various experimental conditions where made with negative (HWB unexposed to other tissues or endotoxin; Fig. 2c) and positive (HWB exposed to endotoxin; Fig. 2e) controls.

In a separate study to confirm findings using a different marker of immune activation, HWB from the investigator was employed. HWB was directly exposed to phytohemmaggultin (20 μg/ml) or freeze/thaw damaged EC or KC, or a combination of such cells in which the exposure concentration was 40,000 cells/ml. Following 24 h of incubation under rocking conditions, duplicate 100 μl HWB samples were treated, as prescribed by the vendor (Coulter, Inc.; Miami, FL) with antibody cocktails that permitted determination of lymphocyte subtype and expression of the cell surface marker, cluster of differentiation (CD) 69. Presence of this marker was indicative of a proliferative response following immune activation.

RESULTS

When permeable membranes separated HWB, EC and KC, expression of IL-4 or IL-12 by HWB (Fig. 3) over the 48 h test period was more similar to the negative (Fig. 2c) than positive (Fig. 2e) control. Similar findings (Fig. 4) were noted for the *in vivo*-like experimental condition (Fig. 2b) when cytokine expression was examined for intact EC

exposed to HWB, while a 0.4μ membrane separated the keratinocytes. Findings (data not shown) did not change when HWB was exposed directly to intact EC or KC (Fig. 2a). When a combination of soluble mediators of freeze/thaw-damaged EC and KC where exposed to HWB via the large pores of the 3.0μ membrane (Fig. 2d), IL-4 or IL-12 expression was also not stimulated (Fig. 5). As would be expected, when experimental conditions were the same (Fig. 2d), but a smaller pore (0.4μ) membrane employed, a negative response was recorded (data not shown). Negative findings were also noted when soluble mediators of freeze/thaw-damaged EC or KC were separately exposed to HWB via the 3.0μ membrane or when the smaller pore (0.4μ) sized membrane was employed (Fig. 2d; data not shown). Finally, intact EC or KC exposed to unsupplemented culture media (Fig. 2a) did not induce expression of IL-4 or IL-12 (data not shown)

As illustrated in the C2 quadrates of the flow cytometric histograms for CD69 expression by CD4 T-helper lymphocytes, direct HWB exposure to a combination of freeze/thaw-damaged EC and KC resulted in little or no CD69 expression, when compared to the expression induced by exposure to phytohemagglutinin (Fig. 6). Findings were similar for CD8 T-cytotoxic/suppressor lymphocytes and CD19 B-cell lymphocytes (data not shown). In addition, when an analysis for CD69 expression by these various lymphocyte subpopulations following separate exposures to either freeze/thaw-damaged EC or KC, findings were unchanged (data not shown).

DISCUSSION

Participation of the immune system in the tissue injury cascade associated with exposure to environmental extremes is a significant pathophysiological feature (1-7). Models are required to appropriately study the contributions of the immune system in such forms of injury. The present study evaluated an approach to model local immune responses induced by tissue injury and necrosis as seen after freeze/thaw or frostbite damage. The model was based on the primus immunologically distinct tissues could be

employed, if no immune system induction occurred when histocompatibility complexes on the surfaces of intact tissues where physically separated from HWB by permeable membranes, but such induction did occur when soluble mediators from freeze/thaw damaged cells (i.e., tissue necrosis) traversed the small pores (0.4μ) of the membrane.

The lack of an IL-4 or IL-12 response by HWB, EC and KC physically separated from each other by permeable membranes (Fig. 3) indicated isolation of the different histocompatibility complexes on the surface of these intact, immunologically distinct tissues prevented an immune response. Moreover, passage of soluble mediators generated by these separated, intact tissues through the pores of the membrane did not induce a reaction. Together these findings suggested immunologically distinct tissues could be employed in this model system. However, only a weak or no immune response was recorded when HWB was directly exposed to intact EC, while a 0.4μ membrane separated the KC (Figs. 4), nor when intact EC or KC were directly exposed to HWB in the absence of any other tissue (data not shown). Similarly, soluble mediators from freeze/thaw damaged EC and KC exposed to HWB did not induce an IL-4 or IL-12 response (Fig. 5).

To address the possibility of missing signs of immune activation by focusing on down-stream cytokine responses, studies were conducted to examine the immune cell proliferative response, a primary step in immune activation. CD69 is an early cell surface marker that correlates closely with immune cell proliferation (25-27). As such, CD69 expression serves as an excellent marker to indicate the initiation of immune activation. CD69 expression was not noted for CD4 T-helper lymphocytes exposed to intact or freeze/thaw-damaged EC and KC (Fig. 6). Similar findings were noted for other lymphocyte subpopulations (data not shown)

Though an exhaustive analysis of all possible indicators of immune activation has not been conducted in this study, early (CD69 expression) and down stream (cytokines) immune system markers have been explored and the findings suggested the experimental design employed in this *in vitro* model would not support an immune response either by tissue necrosis or an adverse histocompatibility reaction. Additional

subjects would need to be studied to come to a definitive conclusion that an IL-4 response was not induced by freeze/thaw-damaged tissues (Fig. 5), since the endotoxin positive control did not result in a pronounced production of IL-4. However, in this model a cellular (IL-12) rather than a humoral (IL-4) immune reaction was to be expected. Since IL-12 and CD69 expression suggested a lack of an immune response, it did not seem warranted to expend limited In-house Laboratory Independent Research funds to further pursue the IL-4 question.

Comparison with *in vitro* systems that model allograft tissue rejection (18-20) may provide an explanation for the lack of success in the present experimental design. The allograft rejection models employ human skin explants that retain features, such as the presence of Langerhans cells, specialized antigen presenting or dendritic cells located in the epidermis. In addition, such explants may also retain aspects of the lymph system. The current model lacked these features, which may be essential to mounting an immune response leading to cytokine expression and/or immune cell proliferation. Moreover, cultured cells, as opposed to fresh tissue explants may over time experience a reduction in their antigenicity.

CONCLUSIONS

Using immunologically distinct donors for HWB, EC and KC, immune activation under *in vitro* conditions via a necrotic or histocompatibility pathway did not materialize. This conclusion supports findings that antigens that do not reach specialized dendritic or antigen presenting cells, as well as organized lymphatic tissues fail to induce immune responses (28).

RECOMMENDATIONS

Models that permit study of local inflammatory responses associated with tissue damage following exposure to environmental extremes would greatly advance understanding the pathophysiology of the injury cascade. Though the present design was unsuccessful, *in vitro* systems offer a level of control over experimental parameters

and access to local inflammatory environments that would be extremely difficult to replicate in an animal or human model. As such, further work to resolve deficiencies in this type of modeling is warranted. It is recommend the present model might be improved by enriching the HWB with dendritic cells. Normal blood levels for dendritic cells are quite low. Presently there are commercially available sources for dendritic cells. Moreover, there are techniques to select for circulating dendritic cells. Perhaps supplementing HWB with homologous dendritic cells would allow this *in vitro* system to respond to the antigenic signals associated with tissue necrosis to permit the desired immune responses. Though a reduction in the use of research animals is a mandated goal within the Department of Defense, another approach might be to use homologous tissue explants and blood from an appropriate animal in an *in vitro* method to study local inflammatory responses. This might provide the additional immune system features necessary to support responses induced by necrosis and circumvent issues of lower antigenicity perhaps associated with the use of cultured cells.

FIGURE 1. ILLUSTRATION OF THE METHODS EMPLOYED TO PHYSICALLY SEPARATING HUMAN TISSUES, WHILE PERMITTING EXCHANGE OF SOLUBLE MEDIATORS THROUGH THE PORES OF SEMIPERMEABLE MEMBRANES.

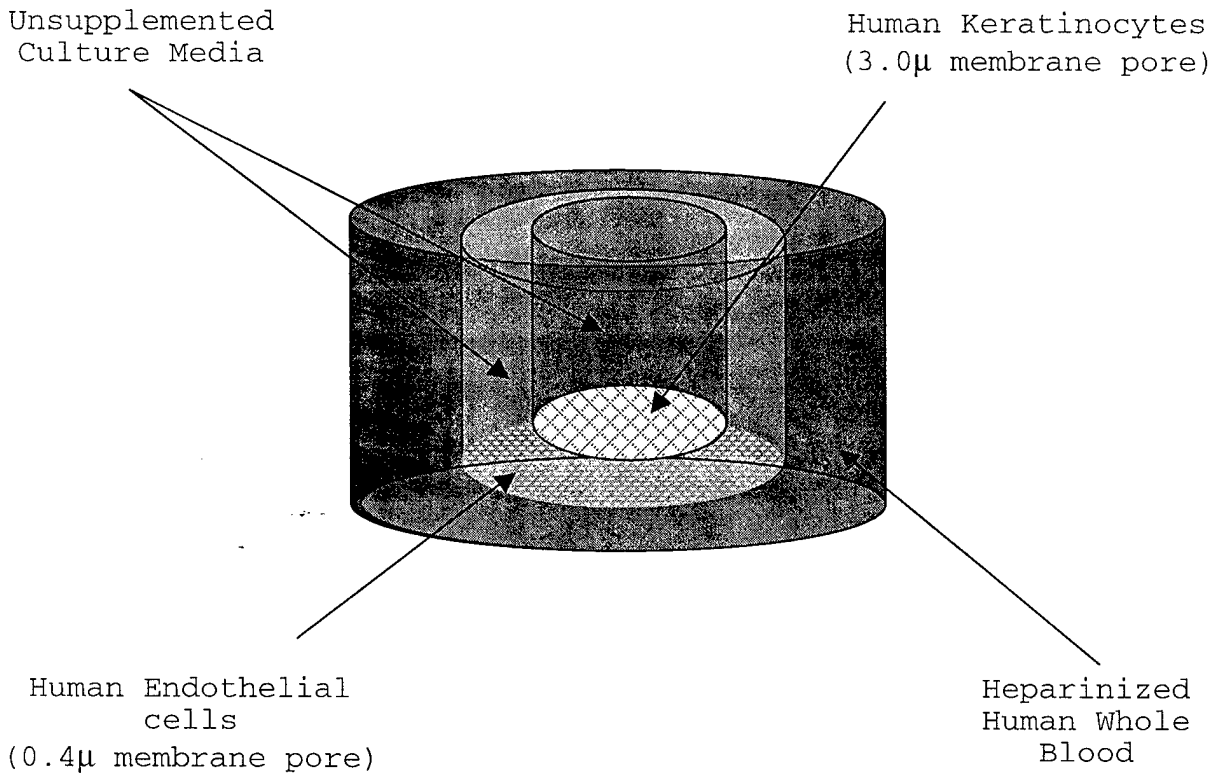
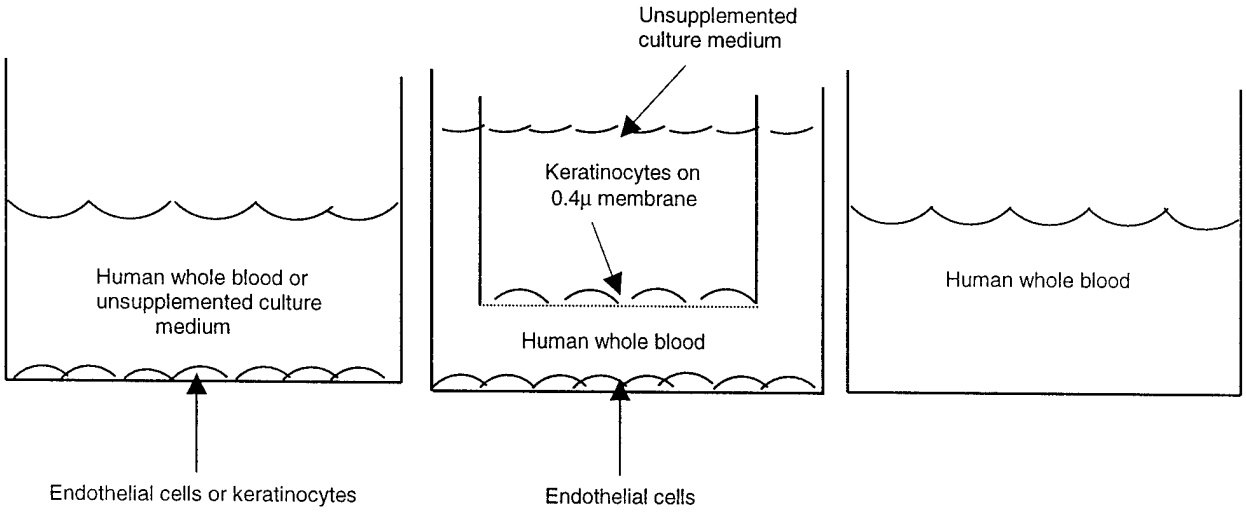


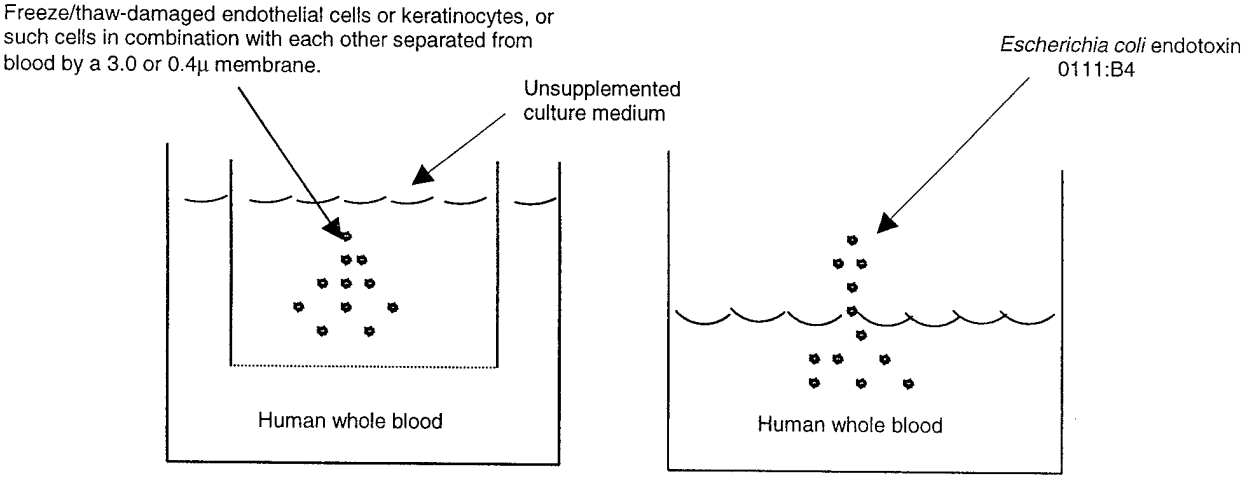
FIGURE 2. ILLUSTRATION OF THE VARIOUS EXPERIMENTAL CONDITIONS TESTED FOR IL-4 OR IL-12 RESPONSES.



2a. Controls for immune activation by endothelial cells or keratinocytes in the presence of human whole blood or culture medium.

2b. *In vivo*-like control for immune activation by endothelial cells exposed to blood, while a 0.4μ membrane separated keratinocytes

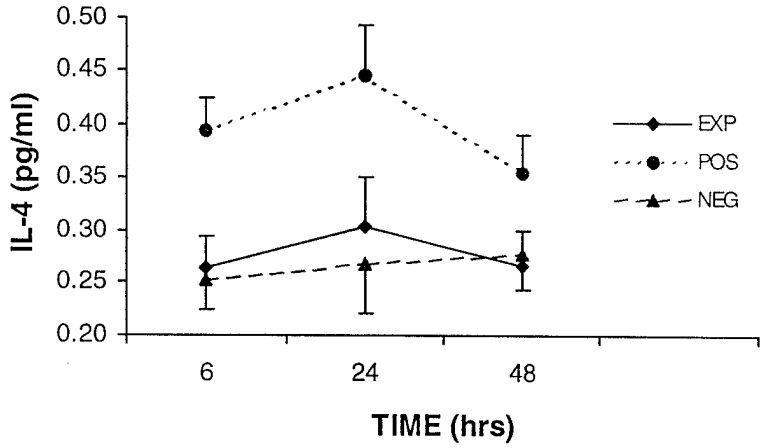
2c. Negative control for immune activation by human whole blood



2d. Controls for immune activation by freeze/thaw-damaged cells.

2e. Positive control for immune activation by human whole blood.

FIGURE 3. IL-4 OR IL-12 EXPRESSION COMPARISONS AMONG POSITIVE CONTROL (POS; FIG. 2E), NEGATIVE CONTROL (NEG; FIG. 2C) AND THE EXPERIMENTAL CONDITION (EXP; FIG. 1) IN WHICH HUMAN WHOLE BLOOD WAS SEPARATED FROM ENDOTHELIAL CELLS OR KERATINOCYTES BY PERMEABLE MEMBRANES.



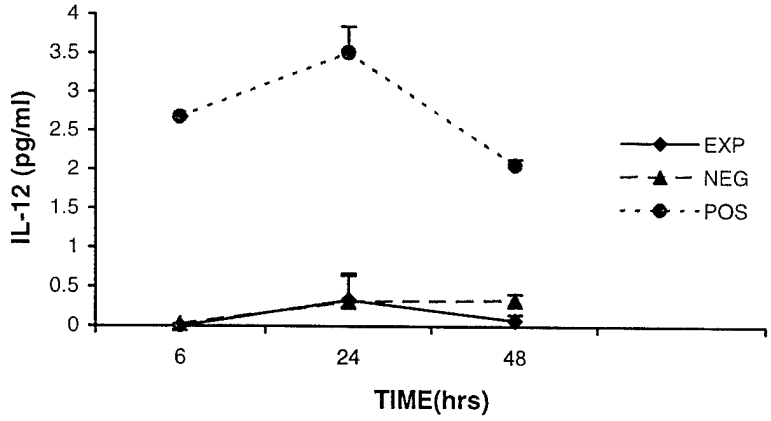
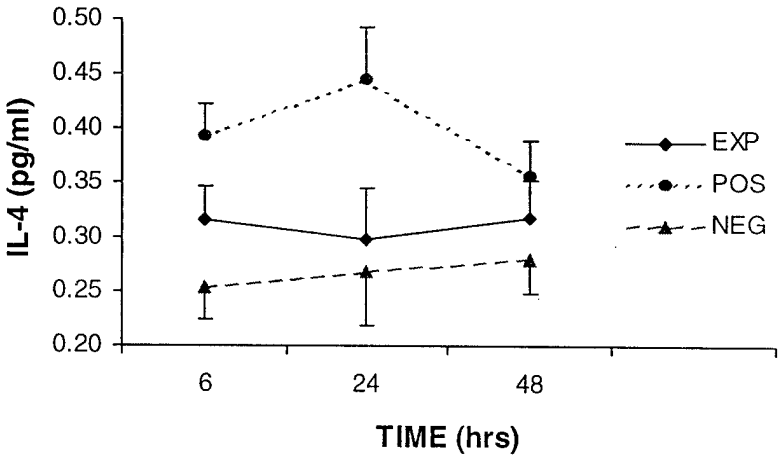


FIGURE 4. IL-4 OR IL-12 EXPRESSION COMPARISONS AMONG POSITVE CONTROL (POS; FIG. 2E), NEGATIVE CONTROL (NEG; FIG. 2C) AND THE EXPERIMENTAL CONDITION (EXP; FIG. 2B) IN WHICH HUMAN WHOLE BLOOD WAS EXPOSED TO INTACT ENDOTHELIAL CELLS, WHILE INTACT KERATINOCYTES WERE SEPARATED BY A 0.4μ MEMBRANE.



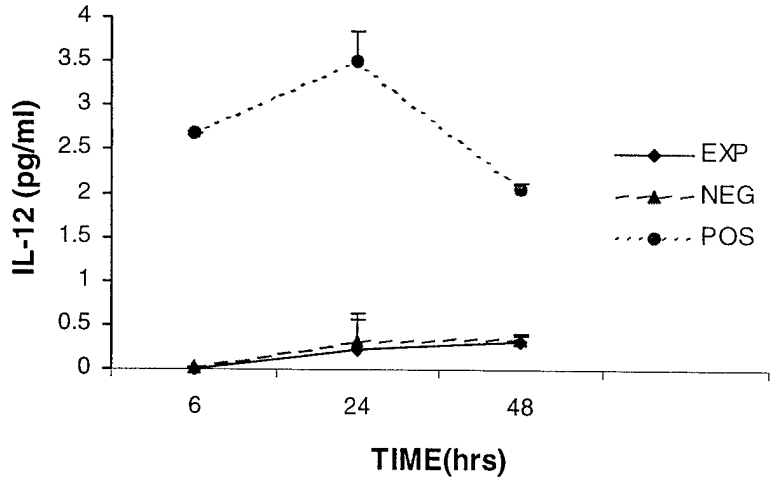
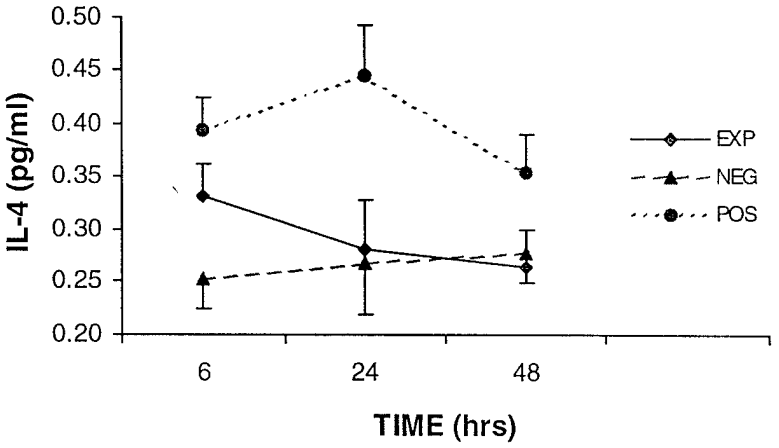


FIGURE 5. IL-4 OR IL-12 EXPRESSION COMPARISONS AMONG POSITVE CONTROL (POS; FIG. 2E), NEGATIVE CONTROL (NEG; FIG. 2C) AND THE EXPERIMENTAL CONDITION (EXP; FIG. 2D) IN WHICH HUMAN WHOLE BLOOD WAS EXPOSED TO FREEZE/THAW-DAMGED ENDOTHELIAL CELLS AND KERATINOCYTES SEPARATED BY A 3.0μ MEMBRANE.



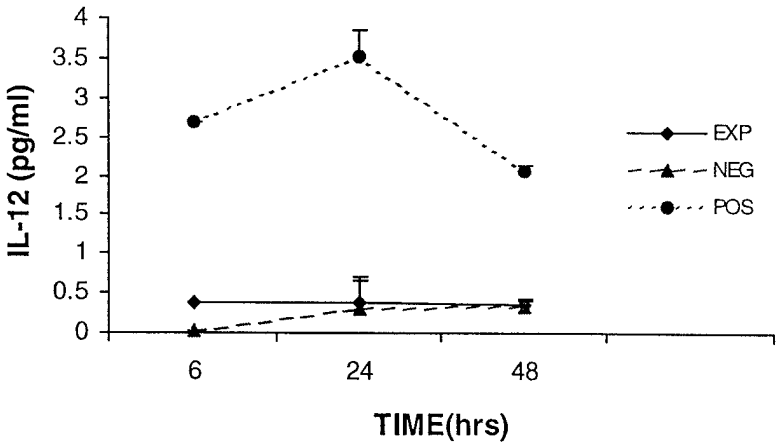
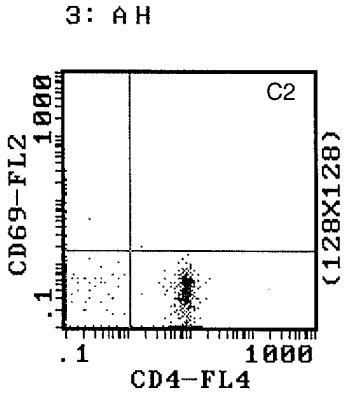
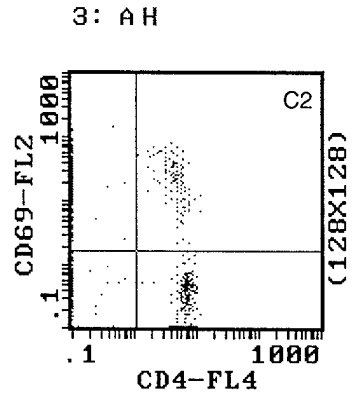


FIGURE 6. FLOW CYTOMETRIC HISTOGRAMS ILLUSTRATING A COMPARISON OF CD69 EXPRESSION BY CD4 T-HELPER LYMPHOCYTES DIRECTLY EXPOSED TO A COMBINATION OF FREEZE/THAW-DAMAGED ENDOTHELIAL CELLS AND KERATINOCYTES (A) OR PHTOHEMAGGLUTININ (B).



A. Human whole blood exposed (24 hrs) to freeze/thaw-damaged endothelial cells and keratinocytes (40,000 cells/ml).



B. Human whole blood exposed (24 hrs) to phytohemagglutinin (20 µg/ml).

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